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A segment of the C-terminal half of the G-protein β1 subunit specifies its interaction with the γ1 subunit

(signal transduction/phospholipase/βγ dimers)

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ABSTRACT **The β and γ subunits of the heterotrimeric guanine nucleotide binding (G protein) act as a dimer and directly regulate various signal transduction pathways. By using cotransfection assays, we tested the ability of several βγ combinations to activate inositol phospholipid-specific phospholipase C (PI-PLC)-β2. Our findings indicate that only βγ combinations that form dimers will activate PI-PLC-β2. Since Gβ1 interacts with Gγ1, while Gβ2 cannot, chimeras between Gβ1 and Gβ2 were used to identify the regions in β1 that determine its specific association with γ1. Our evidence demonstrates that a chimera between β2 and β1 that contains the C-terminal 173 amino acids of β1 can interact and activate PI-PLC-β2 with γ1. Chimeras that contain portions of the β1 C-terminal region display a weaker association with γ1. Furthermore, the contribution of each of these regions depends on the sequence context of each chimeric protein. However, the segment between residues 210 and 293 of β1 consistently plays a critical role in specifying association with γ1.**

Heterotrimeric guanine nucleotide binding proteins (G proteins) transduce signals from cell surface receptors to a variety of effector systems via a guanine nucleotide binding and hydrolysis cycle. The Gα subunits activate second messenger-generating systems (1–3). Furthermore, there is evidence that βγ dimers also have an active role in signal transduction pathways (4–6). Recent efforts have shown that the Gβγ dimer can contribute to the selective coupling of the heterotrimeric G protein to its receptor (7–9) and can regulate various downstream effector systems such as adenylyl cyclase, inositol phospholipid-specific phospholipase C (PI-PLC), the atrial potassium channel, a cytosolic inositol phospholipid 3 kinase, and mitogen-activated protein kinases (10–20). In addition, Gβγ can facilitate the translocation of specific kinases to the membrane (21, 22).

At present, five distinct β subunits have been cloned (23–25), and there are at least seven different Gγ subunits (23, 24, 26). Testing for the interaction between β and γ subunits by using cell-free and transfection systems has indicated that the β1 subunit can interact with both γ1 and γ2 subunits, whereas β2 can interact with γ2 and not with γ1 (27, 28). By using purified recombinant βγ dimers, β1 has been found to dimerize with γ1, γ2, γ3, γ5, and γ7, whereas β2 can dimerize with γ2, γ3, γ5, and γ7 but not with γ1 (29–31). Similar findings came from transfection studies testing the ability of βγ combinations consisting of β1 or β2 with γ1, γ2, or γ5 to activate PI-PLC-β2 (11, 32). The structural basis for the interactions between the β and γ subunits has not been established. Crosslinking experiments show that γ1 can interact with the N terminus of β1 (33). Computer-assisted molecular modeling of the β and γ subunits revealed that their N-terminal regions can form α-helical coiled-coil structures and it was postulated that β and γ dimerize through these regions (34, 35). However, the N-terminal coiled-coil structure cannot account, by itself, for the exceptional stability of βγ dimers (36). Recently, Pronin and Gautam (28) showed that γ1 does not bind β2 but binds to a chimeric β2–β1 protein containing the 59 N-terminal residues of β2, indicating that sequences downstream of residue 59 in β1 contribute to the stability and specificity of the β1γ1 dimer. In the present study, by using the cotransfection assay, we identified the region of the β1 subunit that determines its specific interaction with the γ1 subunit.

MATERIALS AND METHODS

cDNA Constructs and Transient Transfections into COS-7 Cells. The cDNAs were inserted into cytomegalovirus expression vectors as follows: Gβ1 and Gγ2 subunits were cloned into pCDM8.1 (Invitrogen); PLC-β2 was inserted in pMT2; and the Gγ1 mutant γ1C71L with the Cys-71 → Leu substitution was introduced into pCIS. A bacterial β-galactosidase gene in pCIS served as a transfection control. The chimeras between Gβ1 and Gβ2 were constructed by synthesizing each corresponding cDNA portion with the PCR. Both portions of the chimeric cDNA were fused through a common restriction site that was created by introducing silent mutations at the junction sites. The common restriction endonuclease sites created were *Bss*HII, *Hin*dIII, *Sal* I, and *Mun* I at positions corresponding to amino acids (aa) 167, 209, 250, and 293, respectively. Each pair of synthesized fragments was digested with the corresponding restriction enzymes and inserted between the *Cla* I and *Not* I of pCIS. The sequence of the chimeric constructs was verified by restriction enzyme analysis and DNA sequencing using the Dye-Terminator method (Perkin–Elmer ABI) and the 373A automated sequencer of ABI.

COS-7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% (vol/vol) fetal calf serum. Approximately 1×10^5 cells per well were seeded in 12-well plates 1 day before transfection. The total amount of DNA in all transfections was 0.9 μg per well. The amount of each type of DNA in each set of experiments was equal and pCIS encoding β-galactosidase was used to maintain a constant amount of DNA. To each well, 0.9 μg of DNA mixed with 5 μl of LipofectAmine (Bethesda Research Laboratories) in 0.5 ml of Opti-MEM (Bethesda Research Laboratories) was added and 5 h later 0.5 ml of 20% fetal calf serum in DMEM was added to the cells. Cells were assayed for inositol phosphate levels or harvested for protein expression analysis 48 h after transfection.

Analysis of Inositol Phosphate Levels in Intact Cells. One day after transfection, the medium was removed and the cells were washed with phosphate-buffered saline (PBS) and incubated in 0.4 ml of inositol-free DMEM with 10% dialyzed fetal calf serum containing *myo*-[2-^{3}H]inositol (NEN) at 10 μCi/ml (1 Ci = 37 GBq). Twenty-four hours later, the cells were

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Abbreviations: G protein, guanine nucleotide binding protein; PI-PLC, inositol phospholipid-specific phospholipase C.

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washed with PBS, 200 μl of inositol-free medium containing 10 mM LiCl was added, and the cells were incubated for 25 min at 37°C. Each reaction was stopped by adding 200 μl of ice-cold 10% (vol/vol) perchloric acid and 20 μl of phytic acid (20 mg/ml) and incubating the cells on ice for 10 min. Then 200 μl of the supernatant was transferred to a microcentrifuge tube and neutralized with 2 M KOH. After centrifugation, the supernatant was loaded on a 0.5-ml AG1-X8 anion-exchange column (200–400 mesh, formate form, Bio-Rad). The separation was done essentially as described by Berridge *et al.* (37) and 0.5 ml of the eluted material was mixed with 10 ml of BCS scintillation solution (Amersham) and radioactivity was measured. All the data represent duplicate determinations in a single experiment. The error bar expresses the range of duplicate samples. Three additional experiments gave similar results. SDS/PAGE and Western blot analysis were done as described (11).

RESULTS

Gβ1 and Gβ2 share 90% identity in their amino acid sequence, yet Gβ1γ1 activates PI-PLC-β2, while Gβ2γ1 does not. On the other hand, both Gβ1γ2 and Gβ2γ2 dimerize and activate PI-PLC-β2 (11, 31, 32). To identify the region on Gβ1 that determines its specific interaction with Gγ1, the activation of PI-PLC-β2 was used as the assay. A reciprocal series of chimeras between Gβ1 and Gβ2 was generated and the ability of these chimeras to presumably form dimers with Gγ1 and activate PI-PLC-β2 in transfected COS-7 cells was tested. Two chimeras were prepared, Chi1, which encodes the N-terminal 167 aa of Gβ1, and Chi2, which encodes the first 209 aa of Gβ1 (Fig. 1*A*). As a control, we measured the expression levels of transfected Chi1 and Chi2 and of the wild-type β1 and β2 and found that all were expressed at roughly similar levels (Fig. 1*B*). Fig. 1*C* shows the levels of inositol phosphates released by these Gβ subunit constructs when cotransfected with Gγ1 and PI-PLC-β2 constructs. β1γ1 directed a very high level of inositol phosphate release, whereas Chi1γ1 and Chi2γ1 promoted marginal increases and β2γ1 did not direct any increase. Since it is possible that the functional ability of the chimeric proteins to activate PI-PLC-β2 has been altered, an additional control experiment was performed. This was done by cotransfecting Chi1, Chi2, β1, or β2 with γ2 (which can dimerize with β1 and β2 and activate PI-PLC-β2; data not shown) and measuring the levels of inositol phosphates released in the presence of transfected PI-PLC-β2. Fig. 1*D* demonstrates that both chimeras are capable of activating PI-PLC-β2 very well when expressed with γ2. To address the possibility that the chimeras can interact with γ1 but cannot activate PI-PLC-β2, we tested for the direct interaction of the chimeras with γ1 by measuring the translocation of Gβ subunits from the membrane fraction to the cytosol, based on the finding that in transfected cells, γ1C71L, a Gγ1 that cannot be isoprenylated, will shift Gβ subunits that it can dimerize with from the membrane fraction to the cytosol (28, 35). Fig. 1*E* shows that coexpression of Chi2 and γ1C71L had very little or no effect on the cellular distribution of Chi2. As control, we tested the effect of the γ1 mutant on β1 and β2, and as expected, a significant portion of Gβ1 shifted to the cytosol but no shift in Gβ2 was observed. These results indicate that Chi1 and Chi2 interact very weakly, if at all, with Gγ1 and suggest that the N-terminal 209 aa of Gβ1 are not sufficient to confer association with Gγ1.

Next, we tested the reciprocal series of chimeric Gβ2–Gβ1 constructs containing an N-terminal sequence from Gβ2 and a C-terminal sequence from Gβ1. The linear structures of the chimeras are illustrated in Fig. 2*A*. The expression levels of the chimeras and wild-type β1 and β2 were found to be essentially similar (Fig. 2*B*). Transfection of Chi3, which encodes the first 167 aa of Gβ2 and the last 173 aa of Gβ1 directed, a 5-fold

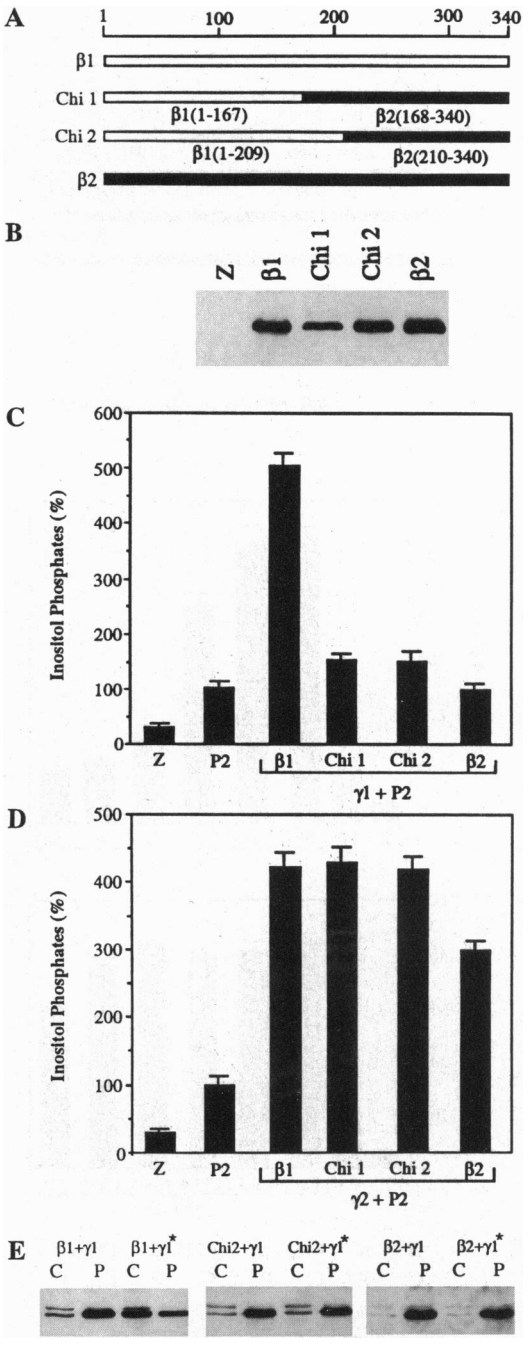

FIG. 1. Structure and expression of the Gβ1–β2 series of chimeras and their effect on PI-PLC-β2 activity in transfected COS-7 cells. (*A*) Linear structures of Gβ1–β2 chimeras. (*B*) Immunoblot analysis of the expression of Gβ1, Chi1, Chi2, and Gβ2 in transfected cells. The Gβ subunits were detected with an antibody directed against the C-terminal 15 aa that are identical in β1 and β2 subunits. (*C* and *D*) The effect of cotransfecting Gβ1, Chi1, Chi2, or Gβ2 with either Gγ1 (*C*) or Gγ2 (*D*) on the activity of PI-PLC-β2 in COS-7 cells. DNAs transfected are indicated for each bar. (*E*) Cellular localization of Gβ1, Chi2, or Gβ2 cotransfected with Gγ1 or with the γ1C71L mutant. Cells were transfected with each cDNA at 0.45 μg per well. Transfected cells were harvested, lysed, and fractionated to cytosolic and particulate fractions (indicated by C and P, respectively). Equal volumes of both fractions were resolved by gel electrophoresis and electroblotted. cDNAs transfected are indicated above the blots, and γ1* represents the γ1C71L mutant. Blots were probed as described in *B*.

increase in the level of inositol phosphates when transfected with Gγ1 and PI-PLC-β2 cDNAs (Fig. 2*C*). This suggests that the C-terminal 173 aa of Gβ1 are sufficient to endow the

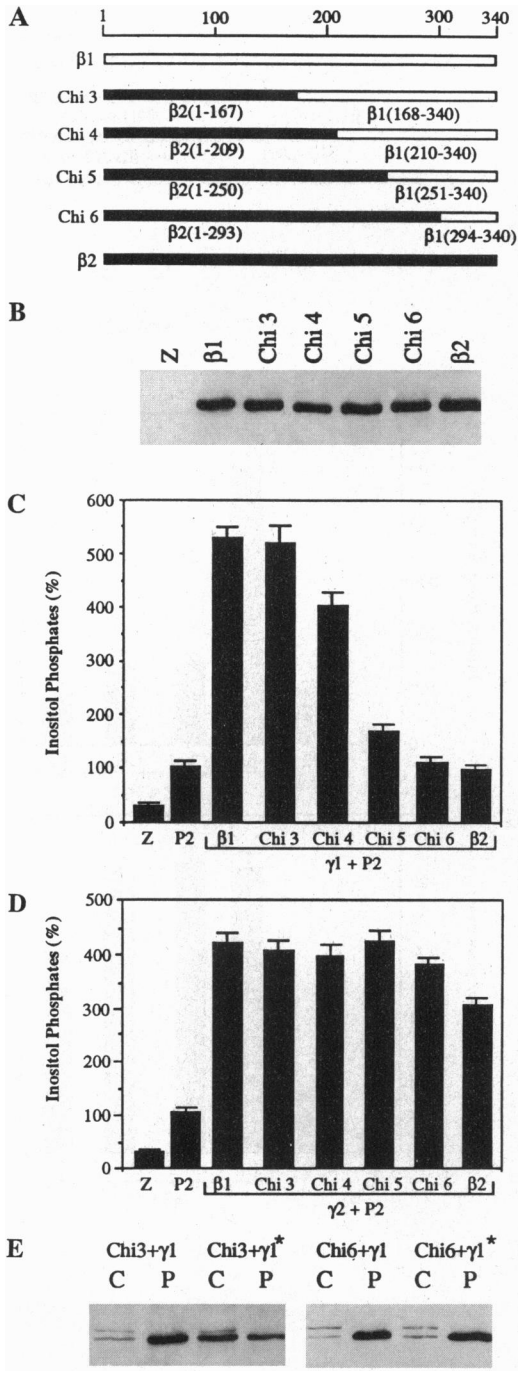

FIG. 2. Structure and expression of Gβ2–β1 series of chimeras and their effect on PI-PLC-β2 activity in transfected COS-7 cells. (*A*) Linear structures of Gβ2–β1 chimeras. (*B*) Immunoblot analysis of the expression of Gβ1, Chi3, Chi4, Chi5, Chi6, and Gβ2 in transfected cells. Blots were probed as in Fig. 1. (*C* and *D*) The effect of cotransfecting Gβ1, Chi3, Chi4, Chi5, Chi6, or Gβ2 with either Gγ1 (*C*) or Gγ2 (*D*) on the activity of PI-PLC-β2 in COS-7 cells. DNAs transfected are indicated for each bar. (*E*) Cellular localization of Chi3 and Chi6 cotransfected with Gγ1 or with γ1C71L. Cells were transfected with each cDNA at 0.45 μg per well. Transfected cells were harvested, lysed, and fractionated to a cytosolic and particulate fractions (indicated by C and P, respectively). Equal volumes of both fractions were resolved by gel electrophoresis and electroblotted. cDNAs transfected are indicated above the blots, and γ1* represents the γ1C71L mutant. Blots were probed as described in *B*.

chimeric protein with the ability to associate with Gγ1. To delineate this region further, we tested additional chimeric constructs containing shorter portions of β1 at their C-terminal ends. Chi4, which harbors 131 residues of Gβ1 at its C-terminal end, was able to promote a 4-fold increase in the level of inositol phosphates. Chi5, which contains the C-terminal 90 residues of Gβ1, directed weak increases in the level of inositol phosphates. Chi6, which encodes only the last 47 aa of Gβ1, hardly raised the level of inositol phosphates. Transfection of chimeras with Gγ2 instead of Gγ1 directed a large increase in the levels of released inositol phosphates (Fig. 2*D*), indicating that the chimeras have retained their ability to activate PI-PLC-β2 provided that they can dimerize. These findings imply that the reduced ability of Chi4, Chi5, and Chi6 to activate PI-PLC-β2 when transfected with Gγ1 is probably due to their reduced affinity for Gγ1. To determine association more directly, we tested the effect of the Gγ1 mutant γ1C71L on the cellular distribution of Chi3 and Chi6 in transfected cells. Fig. 2*E* shows that γ1C71L can translocate Chi3 to the cytosol, whereas γ1C71L has a barely detectable effect on the localization of Chi6. These observations indicate that in our assay system, there is a correlation between the ability to activate PI-PLC-β2 and the ability to dimerize with Gγ1. The ability of Chi4, which contains 131 residues of Gβ1 at its C-terminal end, to activate PI-PLC-β2 by 4-fold indicates that the C-terminal 131 aa are essentially sufficient to confer on the chimeric protein the ability to associate with Gγ1. However, the region between residues 168 and 209 of Gβ1 may also contribute to the interaction with Gγ1 since Chi3 activated PI-PLC-β2 more efficiently than Chi4. The significantly reduced ability of Chi5 and Chi6 to activate PI-PLC-β2 pinpoints the region of Gβ1 between aa 210 and 293, as essential in determining the specific interaction of Gβ1 with Gγ1.

To further characterize the C-terminal region of Gβ1, double chimeric constructs were prepared and tested. This was done by replacing portions of the C-terminal end of Chi3 with the corresponding portions of Gβ2 (Fig. 3*A*). The expression levels of Chi7, Chi8, Chi9, β1, and β2 (Fig. 3*B*) are similar. The inositol phosphate levels measured in cells cotransfected with one of these chimeras, Gγ1, and PI-PLC-β2 are depicted in Fig. 3*C*. Chi7, which harbors the Gβ1 sequence between aa 168 and 209, did not direct an increase in level of inositol phosphates. Chi8, which contains residues 168–250 of Gβ1, increased the level of inositol phosphates by 2-fold. An increase of almost 4-fold in the levels of inositol phosphates was observed with Chi9, which contains the Gβ1 sequence from aa 168 to 293. When Gγ1 was replaced with Gγ2 in transfections, all of the chimeras retained their ability to activate PI-PLC-β2 (Fig. 3*D*). These findings demonstrate that Chi7 cannot activate PI-PLC-β2 when transfected with Gγ1 and indicate that aa 168–209 of Gβ1 are not sufficient for interaction with Gγ1. The ability of Chi8 and Chi9 to activate PI-PLC-β2 implies that the region of Gβ1 between residues 210 and 293 contains elements that additively contribute to the specific interaction of Gβ1 with Gγ1. This finding is consistent with the previous result showing that chimeras lacking this region (Chi5 and Chi6) interact very weakly with γ1 (Fig. 2).

An additional two double chimeric constructs derived from Chi4 were prepared and tested to further evaluate the contribution of the region between residues 210 and 293 of β1; the linear structures of the chimeras are shown (Fig. 4*A*). Transfection of Chi10, which encodes aa 210–250 of Gβ1, directed a marginal increase in the level of inositol phosphates when transfected with Gγ1 and PI-PLC-β2 cDNAs (Fig. 4*C*). Chi11, which harbors residues 210–293 of Gβ1, when transfected with Gγ1 induced a 2-fold increase in the level of inositol phosphates released in transfected cells. Both chimeras were able to activate PI-PLC-β2 when transfected with Gγ2 (Fig. 4*D*). These observations suggest that aa 210–250 are not sufficient to confer on the chimera the ability to associate with Gγ1, whereas a larger portion of Gβ1, including aa 210–293, is sufficient to specify dimerization with Gγ1.

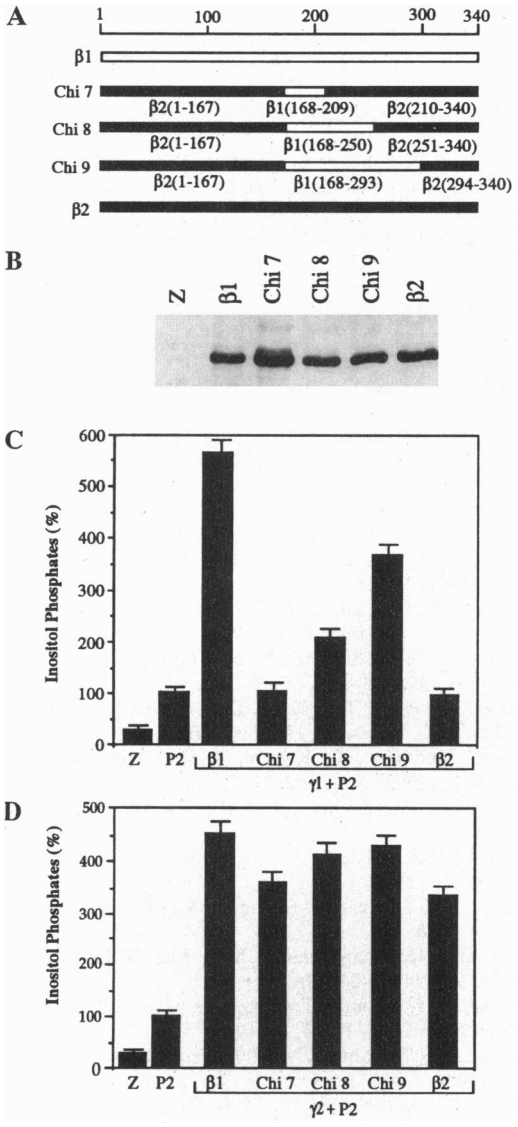

FIG. 3. Structure and expression of Gβ2(167)–β1–β2 series of chimeras and their effect on PI-PLC-β2 activity in transfected COS-7 cells. (*A*) Linear structures of Gβ2(167)–β1–β2 chimeras. (*B*) Immunoblot analysis of the expression of Gβ1, Chi7, Chi8, Chi9, and Gβ2 in transfected cells. Blots were probed as in Fig. 1. (*C* and *D*) The effect of cotransfecting Gβ1, Chi7, Chi8, Chi9, or Gβ2 with either Gγ1 (*C*) or Gγ2 (*D*) on the activity of PI-PLC-β2 in COS-7 cells. DNAs transfected are indicated for each bar. Transfection and analysis of [^{3}H]inositol phosphate levels were as described above.

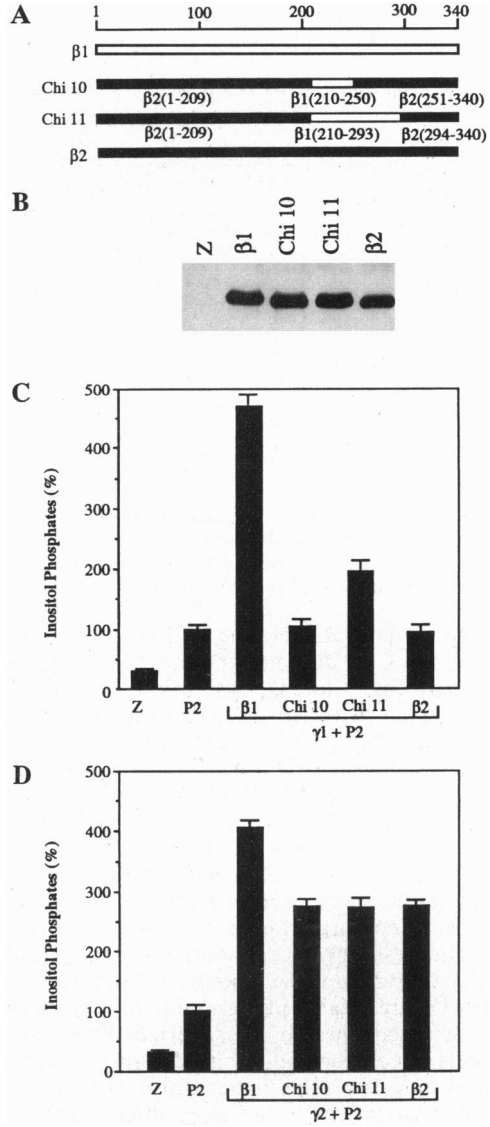

FIG. 4. Structure and expression of Gβ2(210)–β1–β2 series of chimeras and their effect on PI-PLC-β2 activity in transfected COS-7 cells. (*A*) Linear structures of Gβ2(210)–β1–β2 chimeras. (*B*) Immunoblot analysis of the expression of Gβ1, Chi10, Chi11, and Gβ2 in transfected cells. Blots were probed as in Fig. 1. (*C* and *D*) The effect of cotransfecting Gβ1, Chi10, Chi11, or Gβ2 with either Gγ1 (*C*) or Gγ2 (*D*) on the activity of PI-PLC-β2 in COS-7 cells. DNAs transfected are indicated for each bar.

DISCUSSION

Testing the ability of a series of chimeras between β1 and β2 to dimerize with γ1 and activate PI-PLC-β2 has demonstrated that regions in the C-terminal half of β1 determine its association with γ1. Although the N-terminal part of β1 has been implicated in its association with the γ subunit, we found that chimeras harboring up to 209 aa from the N-terminal end of β1 interacted very weakly, if at all, with γ1. On the other hand, since all the chimeras and β1 and β2 were able to dimerize with γ2, it is tempting to postulate that the N terminus although associated with the γ subunit is not sufficient to determine specificity for γ1. Our assay for dimerization was based on the observation that only βγ dimers can activate PI-PLC-β2. All chimeras were able to activate PI-PLC-β2 with γ2. Furthermore, we tested the association of several chimeric proteins with γ1 in a direct assay and found a correlation between the ability of the chimeras to dimerize with γ1 and their ability with γ1 to activate PI-PLC-β2. Our results clearly indicated that a chimeric protein of β2–β1 harboring the C-terminal 173 aa of β1 (Chi3, Fig. 2) with γ1 was able to activate PI-PLC-β2. Chimeras that contain shorter portions of β1 in their C terminus directed less activation of PI-PLC-β2. Chi4, which contains only the last 131 residues of β1, was able to direct ≈80% of the activation of PI-PLC-β2. The contributions of aa 210–250 and 251–293 to the association with γ1 are important since chimeras lacking these sequences (Chi5 and Chi6, Fig. 2) directed very little activation of PI-PLC-β2 when expressed with γ1. As seen in Fig. 5, β1 differs from β2 in the region of aa 168–293 by 18 aa that are scattered along the sequence. Our results show that residues 168–209 of β1 cannot confer association with γ1 (Chi7, Fig. 3). A chimera (Chi8) containing a larger portion of β1, aa 168–250, showed 40% activation, whereas a chimera harboring residues 168–293 directed 65% of the activation observed with β1 (Chi9, Fig. 3). These findings suggest that sequences in β1 between residues 210 and

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Gβ1 MSELDQLRQEAEQLKNQIRDARKACADATLSQITNNIDPVGRIQMRTRRT 50
Gβ2 ....E.........R..........G.S..T...AGL..............  50

Gβ1 LRGHLAKIYAMHWGTDSRLLVSASQDGKLIIWDSYTTNKVHAIPLRSSWV 100
Gβ2 ....................................................  100

Gβ1 MTCAYAPSGNYVACGGLDNICSIYNLKTREGNVRVSRELAGHTGYLSCCR 150
Gβ2 ..........F.............S..............P..........  150
                   167|168
Gβ1 FLDDNQIVTSSGDTTCALWDIETGQQTTTFTGHTGDVMSLSLAPDTRLFV 200
Gβ2 .......I...................VG.A..S...........G.T..  200
          209|210                                250|251
Gβ1 SGACDASAKLWDVREGMCRQTFTGHESDINAICFFPNGNAFATGSDDATC 250
Gβ2 .......I......DS......I........VA.....Y..T........  250
                                              293|294
Gβ1 RLFDLRADQELMTYSHDNIICGITSVSFSKSGRLLLAGYDDFNCNVWDAL 300
Gβ2 ...........LM.............A..R...............I...M  300

Gβ1 KADRAGVLAGHDNRVSCLGVTDDGMAVATGSWDSFLKIWN            340
Gβ2 .G.....................................           340
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FIG. 5. Amino acid sequence comparison between Gβ1 and Gβ2. Both sequences are aligned. Dots represent amino acid identity. The arrows indicate the junction sites of the various chimeras and the numbers mark the amino acid positions of the junction sites.

293 can confer on the chimera the ability to associate with γ1. The finding that Chi9 does not activate PI-PLC-β2 as well as Chi3 (compare Fig. 3 to Fig. 2) indicates that the sequence outside the region of aa 210–293 region stabilizes this region and enhances its activity. Although, the region of aa 210–250 was found to contribute to the association with γ1, this region by itself cannot confer on Chi10 association with γ1. However, even Chi11, which includes the entire region but lacks the rest of the β1 context, was able with γ1 to direct only 40% of the activation of PI-PLC-β2 found with β1γ1, supporting the suggestion that the segment from aa 210 to 293 is stabilized in a specific active conformation in the context of the other β1 sequences. Interestingly, several mutations in the β subunit of yeast (ste4) that disrupted its ability to interact with the γ subunit (ste18) are located in this region (38). Some stretches of amino acid sequence in the C-terminal half of β1 that determine its association with γ1 may interact directly with γ1, while others may determine the conformation of the protein and contribute indirectly to the association with γ1.

Note Added in Proof. While this manuscript was under review, a paper by Garritsen and Simonds (39) that arrived at similiar conclusions by using different assay procedures appeared.

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